



The effect of water activity on thermal resistance of *Salmonella* in chocolate products with different fat contents

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ABSTRACT

Several instances of *Salmonella* outbreaks have been documented in association with chocolates. Conching, an important chocolate manufacturing step, may be designed for control of *Salmonella* if proper product temperature is maintained for sufficient time. A better understanding of thermal inactivation kinetics of *Salmonella* in different chocolate products is, however, highly desirable in evaluating the effectiveness of an existing conching process for *Salmonella* reduction or assessing the need for modification of manufacturing operations. Our previous study on milk chocolate (41.8% fat content) noted that a_w and temperature are two key factors influencing *Salmonella* inactivation in a thermal process. In this research we studied the effect of different fat levels on *Salmonella* inactivation in chocolates at 80 °C. White and dark chocolates (fat content: 53.0% and 74.3%) were used in this study. The samples were conditioned to three water activity (a_w) levels, 0.23, 0.33, and 0.43, at room temperature. The changes of a_w in these samples were quantified when heated to 70 °C and 80 °C to reflect the real relative humidity conditions during the process. The data indicated an increase in the a_w in the chocolate products, correlating with elevated temperatures. The thermal inactivation tests showed that D -values (time to achieve one log reduction at a fixed temperature) of *Salmonella* were between 33.9 and 46.5 min when treated at 80 °C, the corresponding $a_{w, 80\text{ °C}}$ were 0.29 and 0.28 in white and dark chocolate samples, respectively. Our data suggests that the higher fat content contributed to a less increase in a_w , leading to a higher heat tolerance of bacteria in the chocolates. The research results may offer useful insights for the chocolate manufacturers to utilize conching process for the pathogen control of chocolate products made with different recipes.

1. Introduction

Multiple *Salmonella* outbreaks incidents involving chocolate products have occurred globally, spanning from 1973 to 2022 (Werber et al., 2005; World Health Organization, 2022). Our recent research demonstrates the ability of *Salmonella* to endure within chocolate products throughout their typical shelf life (Sun, Xie, Zhou, et al., 2023). There are three main categories of chocolate products in the market, namely milk, white, and dark chocolate. Different formulations are used in manufacturing these products, some of the ingredients are susceptible to microbial contamination. Regardless of the ultimate products, conching is the final heat treatment step before packaging. The reported outbreaks associated with chocolates suggest that the current conching practices may not be adequate for pathogen control. The conching parameters applied in the production of commercial chocolates are subject to

variation, contingent on the specific chocolate products being processed. Typically, conching for dark chocolate occurs at temperatures between 70 and 80 °C (Wollgast & Anklam, 2000), while lower temperatures, approximately 50–60 °C, are favored for milk chocolate (Urbańska et al., 2021). Traditional conching processes primarily aim to control particle dimension, enhance mouthfeel, and regulate moisture content (MC), rather than to eliminate bacteria (Augusto & Bolini, 2022). To ensure production of safe chocolate products, it is imperative to assess if the current conching operations are able to achieve adequate *Salmonella* reduction and, more importantly, if there is a need for modifications to the conching conditions or additional thermal processing. Such assessments should be grounded in sound scientific data pertaining to the thermal resistance (D -value) of *Salmonella* in various chocolate products.

Water activity (a_w) is an important measure of available free water for microbial growth in food matrices. It also greatly influences survival

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and thermal tolerance of *Salmonella* in low moisture foods, including chocolates and high sugar content products (Alshammari et al., 2020; Sun, Xie, Zhou, et al., 2023; Syamaladevi et al., 2016; Xie, Yang, et al., 2021). The value of water activity in a food product depends on its composition, and changes with temperature (Morasi et al., 2022; Xu et al., 2019; Yang, Guan, et al., 2020). Predictably assessing bacterial *D*-value hinges on a comprehensive grasp of the interplay among treatment temperature, MC, a_w , and the influence of chocolate product composition, including its fat content. Cocoa butter is the primary component responsible for the elevated fat content in chocolates (Ewens et al., 2021). Normally, chocolate fat content falls within the range of 29%–50% (USDA, 2018; USDA, 2019). Six polymorphic forms (I–VI) were found in cocoa butter, each with a different melting point (16–36 °C). The form V cocoa butter (melts between 34 and 35 °C) not only imparts a satisfying snap, a glossy appearance, and a smooth mouthfeel, but also bestows robust resistance to commercial chocolate fat blooming phenomenon (Ghazani & Marangoni, 2021). At room temperature, chocolates usually maintain a a_w between 0.30 and 0.50 (Żyzelewicz et al., 2012), while the a_w of unfinished product in a conventional conching process changes continuously as moisture losses to the ambient (Toker et al., 2019).

This study was designed to achieve two primary objectives: 1) investigate the correlation between MC and a_w in chocolate featuring distinct formulations when subjected to elevated temperatures. 2) determine *D*-value of *Salmonella* in three different chocolate products that were preconditioned to three a_w levels (i.e., 0.23, 0.33, and 0.43) at room temperature and consequently heated to and held at 80 °C.

We selected *Salmonella* Enteritidis PT30 for this research because of its association with multiple contamination incidents in low-moisture food products, including almonds, and chocolate products (Santillana Farakos et al., 2017; D'aoust, 1977). Notably, almonds and sugar are pivotal components within chocolate products. Consequently, we used the same strain to explore the heat tolerance of *Salmonella* in a range of chocolates (solid).

2. Materials and methods

2.1. Preparation and physicochemical characteristics of chocolate samples

The study utilized the raw materials from a local chocolate ingredients supplier (Pullman, WA, USA). These ingredients were used to make both dark and white chocolate samples in accordance with established recipes as documented in previous studies (Jasson et al., 2011; Tamminga et al., 1976; Vercet, 2003). The preparation of milk, white, and dark chocolate samples involved a series of standardized steps. The raw materials were combined in autoclaved 500 ml beakers followed by heating in water at a controlled temperature of 80 °C for 2 h (Toker et al., 2019). Subsequently, the mixtures were left to solidify within sterilized petri dishes at 21 ± 2 °C. The physicochemical properties analysis of the test samples was undertaken by Silliker, Inc (Northern California Laboratory in Salida, CA, USA). The analytical methods used following the procedures outlined by the AOAC (Association of Official Analytical Collaboration).

2.2. Sample conditioning at elevated water activities

A sterilized grater was used to grind the solidified chocolate samples into chocolate flakes. The flakes were then subjected to a conditioning phase within a controlled environment. This conditioning was carried out at 21 ± 2 °C between three and seven days. For objective one, five separate humidity jars were used, each containing various concentrated salt solutions (i.e., CH₃COOK-22.5%, MgCl₂-32.8%, K₂CO₃-43.2%, Mg(NO₃)₂-52.9%, NaNO₂-65.8%) to achieve stable a_w levels of 0.23, 0.33, 0.43, 0.53, and 0.66, respectively, at 21 ± 2 °C, following the method as detailed in Xie, Cheng, et al. (2021). The a_w of the conditioned samples

at room temperature were measured using an Aqualab Model Series 3 TE Water Activity Meter (METER Group Inc., Pullman, WA). The moisture content (MC) of the conditioned chocolate samples was calculated from the increase in weight resulting from the conditioning procedure and the initial MC. Each experiment was conducted three times independently.

2.3. Determination of water activity in chocolates at elevated temperatures

In determining the water activity (a_w) of the chocolate samples exceeding 60 °C, 8 g of chocolate sample, which had undergone equilibration in the preconditioning described above, were moved into specialized high-temperature test aluminum cells developed at Washington State University. A detailed description of the test cells is provided in Tadapaneni et al. (2017). These cells were sealed tightly and heated in a water bath to pre-set temperatures. A centrally positioned and pre-calibrated sensors (Honeywell HumidIcon™ HIH8120 sensor, Morristown, NJ, USA) were used to accurately measure the temperature and relative humidity (RH) within the test cells during heating and equilibration. Measurement data were recorded every minute. To confirm the attainment of an equilibrium state, at least ten sustained RH values (each sustained for 10 min) were necessary. The measured values of the RH at equilibrium were recorded as the water activities of the samples corresponding to the measured temperatures (Tadapaneni et al. (2017)). Each experiment was conducted three times independently.

2.4. Modeling a_w change in chocolates with temperature

The Clausius-Clapeyron equation (CCE) was used to model water activity change (a_w) with temperature for samples heated at a fixed MC (Tadapaneni et al., 2017):

$$a_{w2} = a_{w1} \exp \left(\frac{q_{st}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \right) \quad (1)$$

where, a_{w1} and a_{w2} represent the a_w of the samples at two different temperatures T_1 and T_2 in Kelvin (K), respectively. The value of q_{st} (kJ/mol) represents the net isosteric heat of sorption, it was calculated from the slope of the curve when the data were plotted as logarithm of a_w ($\ln a_w$) against the reciprocal of temperature ($1/T$). Net isosteric heat is the difference between the total enthalpy change for moisture sorption (reflecting the bonding energy between water molecules and food solids) and the latent heat of water vaporization (reflecting the bonding energy among water molecules). R represents the constant for universal gases (8.314×10^{-3} kJ mol⁻¹K⁻¹) and:

$$q_{st} = a \exp(-b * MC) \quad (2)$$

where, MC is moisture content expressed as grams of water per 100 g of dry solids in the tested samples, a (kJ/mol) and b (g dry solid/g H₂O) are constants.

The values of q_{st} at a fixed moisture content (MC) was first obtained by fitting the measured water activities and the corresponding temperatures to Eq. (1). The values of a and b were then obtained using linear regression of $\ln(q_{st})$ vs. MC.

2.5. Bacteria strains

The selection of *S. Enteritidis* PT30 as the pathogen for this research was motivated by its well-established connection to *Salmonella* outbreaks in common low-moisture foods, which notably includes chocolate (Garces-Vega et al., 2019; Whitworth, 2021). The strain was initially isolated from raw almonds and obtained from Dr. Linda Harris at the University of California, Davis. The bacterial culture was persevered in tryptic soy broth (–80 °C) which was enriched with 0.6% (w/v) yeast extract and 20% (v/v) glycerol (TSBYE).

2.6. Culture preparation

To revive the frozen bacteria, two consecutive transfers were performed at a controlled temperature of 37 °C, with each incubation lasting for precisely 24 h. Following this, one mL aliquot inoculum was evenly distributed onto each tryptic soy agar yeast extract (TSAYE) plate (150 mm × 15 mm). These plates were then subjected to a 24-h incubation period. The bacteria were collected with sterile buffered peptone water (BPW). The mixture was subjected to centrifugation at 8000×g for 10 min. Upon completion of the centrifugation, the supernatant was discarded, and the bacterial pellet was rehydrated with 1 mL of BPW. The bacterial suspension reached a concentration of 10~11 log CFU/mL, making it appropriate for subsequent inoculation procedures. In inoculating samples, one mL of the bacterial suspension was added into 10 g of liquefied chocolate (the melting point of chocolate is ~33 °C) in a sample pouch (4 oz Whirl-Pak). The mixture was then gently kneaded for a period of 5 min. Subsequently, 90 g of molten chocolate were introduced into the pouch and manually massaged for 15 min to ensure thorough uniformity. To validate the consistency of the inoculated sample, sub-samples were randomly extracted from five different sites for enumeration. The chocolate that had been inoculated was then allowed to naturally harden in pouches at room temperature of 21 ± 2 °C. The inoculated solid chocolates were distributed onto dishes after shredding into fine pieces and subjected to a seven-day conditioning in a humidity chamber, with the objective of attaining various a_w levels before isothermal treatments.

2.7. Thermal inactivation in TDT cells II

In measuring the D -values of *Salmonella* in the inoculated chocolates with various a_w , the conditioned samples were hermetically sealed in thin Aluminum thermal-death-test cells (TDT cells II) described in details in Jin and Tang (2019a) and immersed in water baths for isothermal treatments.

Aiming at plotting thermal death curve of *Salmonella* accurately under an isothermal condition, the come-up time (CUT), indicating the period needed for the sample temperature to reach that of the water bath (79.5 °C), was assessed by measuring the temperature at the central point of 1-g chocolate samples. The CUT was approximately 1 min for all test samples. Next, 1 g of inoculated and conditioned chocolate was sealed in each of the TDT cells and immersed in an oil bath (Isotemp™ 5150 H24, Fisher Scientific™, PA, USA). The TDT cells II were retrieved at specific time intervals and promptly placed in an ice bath (up to 1 min), following the method detailed in Xie et al. (2022). The population of microbial survival was counted in duplicate at five specified time intervals. All experiments were repeated in triplicate to ensure reproducibility of results independently.

2.8. Bacterial enumeration

A 1-g chocolate sample contained in each TDT cell II was retrieved with a sterile L-shaped spreader, and then placed into 9 mL of preheated sterile BPW at 37 °C. The homogenate was subjected to a 10-fold serial dilution. Suitable serial-dilution were subsequently spread onto TSAYE with 0.05% (w/v) ferric ammonium citrate. These plates were then incubated at 37 °C for 48 h. Colonies showing a distinct dark core with a white perimeter were recorded as *Salmonella* cells.

2.9. Modeling thermal inactivation kinetics

The thermal inactivation data were analyzed using the following logarithmic-linear model (Peleg, 2006):

$$\log N = \log N_0 - t/D \quad (3)$$

where, t is the time of heating at the set temperature (after the come-up

time); N (CFU/g) is microbial count at t ; N_0 (CFU/g) represents the microbial count immediately after the come-up time; D (min) is the time required to reduce the microbial count by 90%. D -value of *S. Enteritidis* PT30 in a chocolate sample was determined by taking the negative reciprocal of the slope obtained from the log-linear regression analysis of the thermal inactivation curve at each tested temperature.

3. Result and discussion

3.1. Values of a_w and q_{st} in different chocolates at elevated temperatures

As shown in Fig. 1, the a_w in all chocolate samples in sealed containers (thus at fixed moisture contents) exhibited an upward trend as the temperature increased. This is due to the positive net isosteric heat (q_{st}) of hydrophilic molecules (Tadapaneni et al., 2017). The bonding energy is a crucial parameter to predict if the a_w changes positively or negatively at elevated temperatures. The extent to which vapor pressure changes with temperature is dictated by the bonding energy between water molecules and sample solids. If the bonding energy is greater than the bonding between water molecules (positive net isosteric heat), then a_w increases with temperature. On the other hand, when the bonding energy is lower than that between water molecules, the a_w decreases as the temperature increases (Tadapaneni et al., 2017; Yang, Guan, et al., 2020). A comparable correlation between temperature and a_w has been observed in various other low moisture foods, including those with high protein content (e.g., whey protein), high carbohydrate content (e.g., wheat flour), high fat content (e.g., almond flour), as reported in Xu et al. (2019). On the contrary, lipid oil exhibits an opposite behavior, the a_w decreases with increased treatment temperature. For instance, the a_w of peanut oil decreased to 0.34 from 0.93 after the treatment temperature increased to 81.0 °C from 21.8 °C (Yang, Xu, et al., 2020).

Fig. 2 illustrates that the value of q_{st} (calculated using Eq. (1)) decreased with increasing moisture content (MC) in all the three chocolate products. Changes of q_{st} values with MC are summarized in Table 2. At an MC of 1.2 (g/100g), the q_{st} was measured at 5.76 kJ/mol for milk chocolate, and this value notably decreased to 0.89 kJ/mol when MC reached 3.9 (g/100g). A similar trend was noted for both dark and white chocolate samples, but q_{st} values were generally lower in products with higher fat contents, particularly at low moisture contents.

Fig. 3 shows the relationships between moisture contents and water activities of milk, white and dark chocolates at 22 and 80 °C. Dark chocolate consistently had lower MC than other chocolate products across all the tested a_w levels at 22 °C. This observation is logically because of a higher cocoa butter content in the dark chocolate (74.3%), as opposed to other chocolate samples (see Table 1). The strong hydrophobic nature of cocoa butter, stemming from its lengthy carbon chain, considerably weakens its interaction with water molecules. Compared to that at 20°C, lower MC values were observed in all the samples at 80°C across the tested water active range..

3.2. Heat tolerance of *Salmonella* as influenced by fat content in chocolates

After conditioning, 8.4 to 8.8 log₁₀ CFU/g populations of *Salmonella* were present in the inoculated chocolate samples as illustrated in Fig. 4. Thermal inactivation of *Salmonella* at 80 °C closely followed the first-order kinetic model in all the tested samples (R^2 ranging from 0.90 to 0.98). The corresponding $D_{80\text{ °C}}$ -values are summarized in Table 3, along with previously obtained data from Sun, Xie, Yang, et al. (2023). In all cases, D -values decreased with sample water activities. It should also be noted in Table 3 that a_w of the three chocolates at the treatment temperature of 80 °C differed from that at room temperature. As discussed earlier, upon heating, a_w increased differently due to the different compositions, which affected the heat tolerance of *Salmonella* in those samples.

Over the tested range of a_w at 80 °C in this study, the $D_{80\text{ °C}}$ -values of

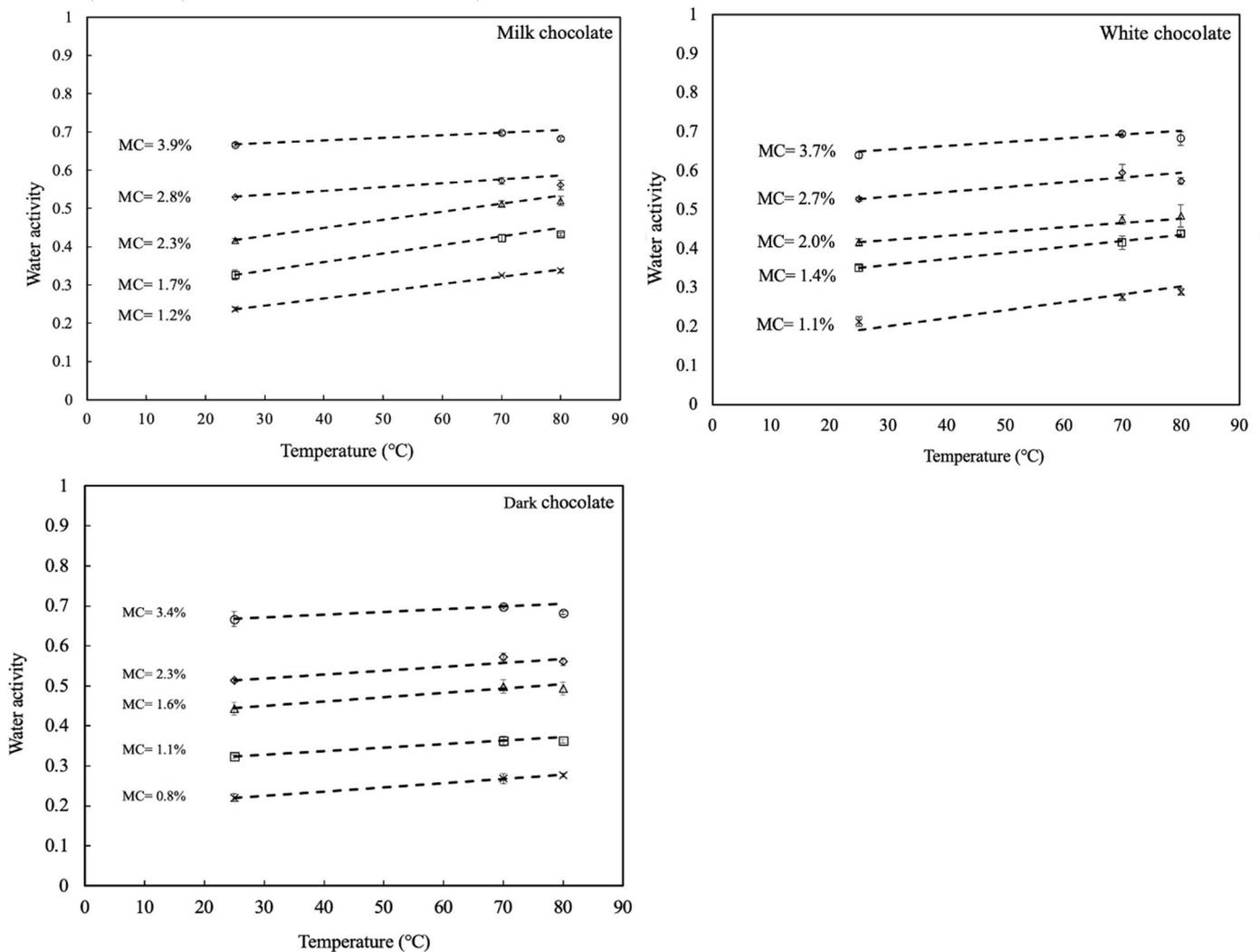


Fig. 1. The relationship between water activity and temperature for three chocolate products. MC is the moisture content of chocolate products (g H₂O/100 g dry solids). The data are means ± SDs (n = 3).

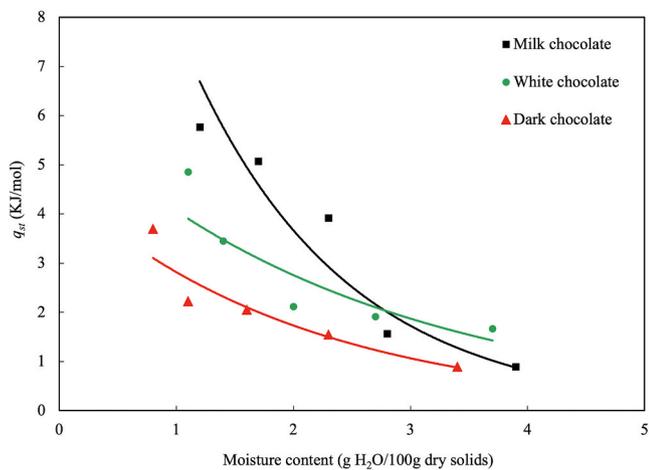


Fig. 2. Net isosteric heat of sorption in three chocolate products as functions of moisture content. Solid lines are fitted lines using exponential-decay model.

Salmonella in chocolate were consistently lower than those of *Salmonella* on SiO₂ and in most of other food matrices reported in the literature (see Fig. 5). This observation indicates that the potential presence of

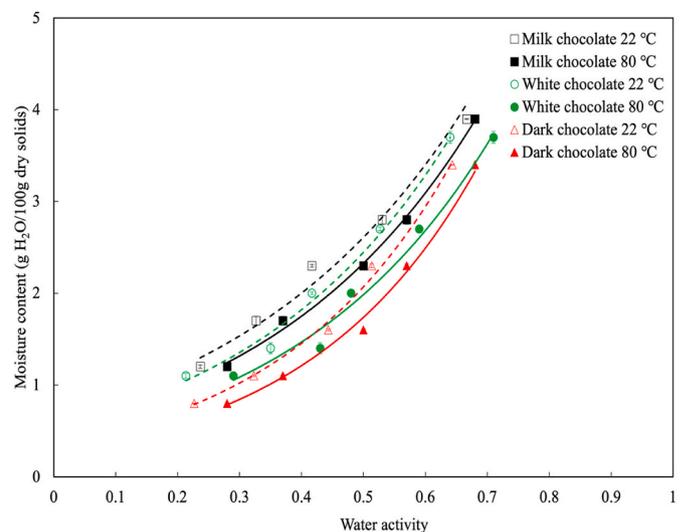


Fig. 3. Sorption isotherms (relationships between moisture content and water activity) for three chocolate products at 22 °C and 80 °C. The data are means ± SDs (n = 3).

Table 1

Proximate composition of chocolate products (n = 2).

Chocolate products	Ash (% w/w)	Carbohydrate (% w/w)	Fat (% w/w)	Moisture (% w/w)	Protein (% w/w)
Milk chocolate	2.75 ± 0.01	40.9 ± 0.2	41.8 ± 0.6	1.44 ± 0.03	13.2 ± 0.4
White chocolate	2.61 ± 0.01	30.1 ± 0.2	53.0 ± 0.2	1.83 ± 0.11	12.4 ± 0.0
Dark chocolate	1.70 ± 0.02	15.2 ± 0.1	74.3 ± 0.1	0.63 ± 0.02	8.17 ± 0.20

Mean ± SD.

Table 2Prediction equation for q_{st} in three chocolate products. q_{st} (kJ/mol) represents the net isosteric heat of sorption, and MC is moisture content (grams of water per 100 grams of dry solid) of the tested samples.

Chocolate products	Prediction equation	Range of MC (g water/100 g dry solids)	Range of q_{st} (kJ/mol)
Milk chocolate	$q_{st} = 16.53 \exp(-0.753 \cdot MC)$	1.2–3.9	5.76–0.89
White chocolate	$q_{st} = 5.97 \exp(-0.387 \cdot MC)$	1.1–3.7	4.85–0.66
Dark chocolate	$q_{st} = 4.58 \exp(-0.486 \cdot MC)$	0.8–3.4	3.69–0.89

antimicrobial compounds may exist in the chocolate products.

Research findings have indicated that the heat tolerance of bacteria in foods with rich fat content, at a given MC, is generally greater compared to foods rich in carbohydrates or proteins (Podolak et al., 2017, pp. 121–148). This phenomenon is attributed to the difference in a_w between fat-rich foods and those abundant in protein or starch (Rahman & Labuza, 2007). The reduced a_w in fat-rich foods contributes to the desiccation of bacteria, subsequently strengthening their resistance to heat (Yang, Xu, et al., 2020). In the current investigation, an enhanced D -value of *Salmonella* in dark chocolate (74.3% fat content) was observed than in white and milk chocolate (53.0% and 41.8% fat content, respectively), although these chocolates were conditioned to equal a_w values measured at ambient temperature. Specifically, the a_w of the dark chocolate of 0.23, 0.33, and 0.43 at room temperature were, respectively increased to 0.28, 0.37, and 0.50 when subjected to heating at 80 °C within sealed containers. This shift in a_w within the chocolate samples and bacterial cells might have resulted in moisture migration, which may either desiccate or hydrate the bacteria until an equilibrium in a_w is achieved, as explained in Xie, Yang, et al. (2021).

Heating can induce permanent alterations in protein structures, including the coalescence of cytoplasmic proteins and the alteration of DNA structure in *S. Enteritidis*, specifically when MC is high (Xu et al., 2020). MC influences the denaturation of proteins and the release of compatible solutes. Under a low moisture environment, these solutes contribute to counterbalance of the osmotic pressure across cell membrane and strengthen the structural stability of the genetic materials, cellular proteins, and ribonucleic particles, allowing them to resist damage caused by heat (Potts, 1994). Consequently, a_w of the food products is a critical determinant affecting bacterial response to heat during the processing of low-moisture foods.

3.3. Implication of the results to chocolate manufacturing

In chocolate manufacturing, conching is a critical step used to enhance the quality of chocolate products. A conching operation involves thorough mixing of all ingredients in rolling mills at temperatures ranging from 50 to 80 °C. Conching can last from several hours to a period of days (Wollgast & Anklam, 2000; Urbańska et al., 2021). It is important to appreciate the correlation between a_w and MC, as illustrated in Fig. 3, highlighting the sensitivity of a_w to changes in MC within

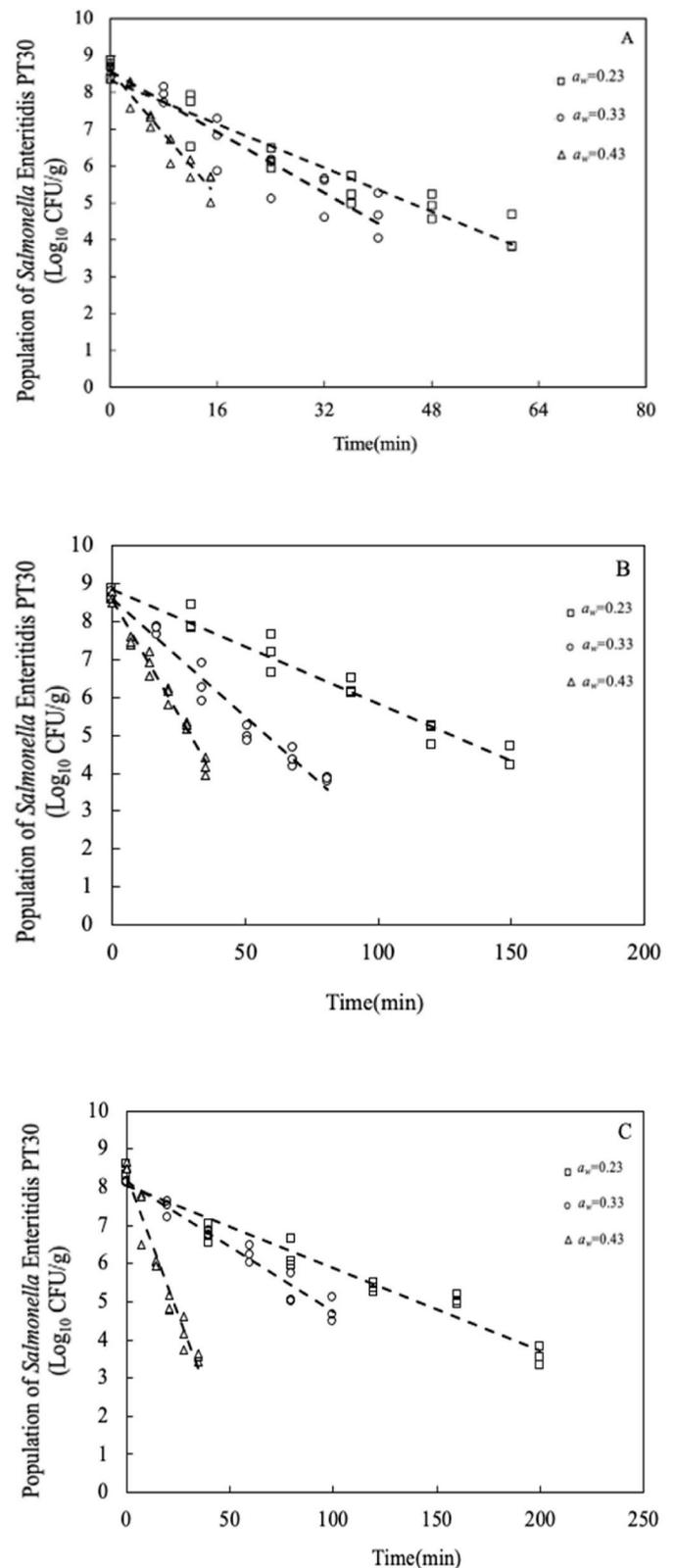


Fig. 4. Thermal inactivation of *S. Enteritidis* PT30 in chocolate products (A: milk chocolate; B: white chocolate; C: dark chocolate) at 80 °C. The initial sample water activities (0.23, 0.33, and 0.43) were measured at 22 °C. Dashed line is fitted line using log-linear model.

Table 3
D-values of *S. Enteritidis* PT30 in three chocolate products at 80 °C (n = 3).

Chocolate products	a_w at 25 °C	a_w at 80 °C	D-value (min)	R^2
Milk chocolate	0.23	0.34	13.6 ± 1.4	0.92
	0.33	0.45	9.1 ± 1.1	0.90
	0.43	0.53	5.2 ± 1.0	0.94
White chocolate	0.23	0.29	33.9 ± 1.4	0.97
	0.33	0.43	17.0 ± 0.9	0.96
	0.43	0.48	8.0 ± 0.2	0.98
Dark chocolate	0.23	0.28	46.5 ± 2.7	0.94
	0.33	0.37	29.0 ± 2.1	0.97
	0.43	0.50	6.7 ± 0.4	0.96

Mean ± SD. Data for milk chocolate are from Sun, Xie, Yang, et al., 2023. The a_w represents the water activity of the tested samples at a fixed temperature.

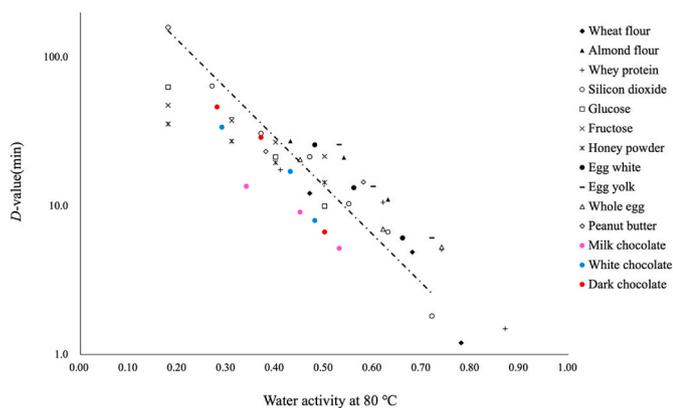


Fig. 5. Comparison of $D_{80\text{ °C}}$ -values of *S. Enteritidis* PT30 in chocolate products with that in other low moisture matrices of different water activities measured at 80 °C (Liu et al., 2018; Xu et al., 2019; Alshammari et al., 2020; Pérez-Reyes et al., 2021; Yang et al., 2022; Sun 2023b). The dashed trend line is for $D_{80\text{ °C}}$ -values of *S. Enteritidis* PT30 inoculated on SiO_2 (from Liu et al. 2018).

chocolate products. In chocolate conching, reduction in both MC and particle size impart a smooth and refined texture (Jolly et al., 2003). It is, therefore, imperative to acknowledge that the decrease in a_w , resulting from evaporation of moisture, could significantly increase the D-value of *Salmonella*, particularly in chocolates with high-fat content. In light of this, it is important to implement vigilant temperature and moisture monitoring to gain a comprehensive understanding of the lethal effects specific conching processes may exert on *Salmonella* within high-fat chocolate products, ensuring both safety and product quality.

4. Conclusion

This research determined the D-values of *S. Enteritidis* PT30 in chocolates and observed a significant influence of product formulation. The impact of formulation (mainly fat content and moisture content) can be explained by the differences in water activity change caused by temperature elevation. This means that the thermal death of *Salmonella* in chocolate is fundamentally governed by the thermal and humidity conditions in the product, just like other many low-moisture foods. The data provided in this article can be used to estimate the lethality of a conching process and provide guidance to help improve process design in achieving desired levels of pathogen control in the chocolate products.

CRedit authorship contribution statement

Sicheng Sun: Writing – original draft, Methodology, Investigation,

Conceptualization. **Ren Yang:** Writing – review & editing, Methodology. **Yuцен Xie:** Writing – review & editing, Methodology. **Mei-Jun Zhu:** Writing – review & editing. **Shyam Sablani:** Writing – review & editing. **Juming Tang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest in this manuscript.

Data availability

Data will be made available on request.

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